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Effects of different brush border membrane vesicle isolation protocols on proteomic analysis of Cry1Ac binding proteins from the midgut of *Helicoverpa armigera*

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Abstract Brush border membrane vesicles (BBMV) isolated from insect midguts have been widely used to study Cry1A binding proteins. Sample preparation is important in two-dimensional electrophoresis (2-DE), so to determine a suitable BBMV preparation method in *Helicoverpa armigera* for 2-DE, we compared three published BBMV preparation methods mostly used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All methods yielded similar types and numbers of binding proteins, but in different quantities. The Abdul-Rauf and Ellar protocol was the best of the three, but had limitations. Sufficient protein quantity is important for research involving limited numbers of insects, such as studies of insect resistance to *Bacillus thuringiensis* in the field. Consequently, we integrated the three BBMV isolation methods into a single protocol that yielded high quantities of BBMV proteins from *H. armigera* larval midguts, which proved suitable for 2-DE analysis.

Key words *Bacillus thuringiensis*, binding protein, brush border membrane vesicles, Cry1Ac, *Helicoverpa armigera*, two-dimensional electrophoresis

Introduction

Bacillus thuringiensis (Bt) has been widely used in pest control as a bioinsecticide or a source of genes encoding insecticidal proteins (e.g. Cry toxins) for transgenic crops (Schnepf *et al*., 1998). For example, Cry1Ac-expressing cotton is protected from attack of lepidopteran pests such as *Helicoverpa armigera*. After Bt-resistant lepidopteran strains were first selected in the laboratory and the first field-selected resistance was reported, different hypotheses have been proposed for the mechanism of insect resistance to Bt, with most concerning alterations to the binding of toxins to receptors in the midgut (Tabashnik *et al*., 1990; Gould *et al*., 1992; Tabashnik, 1994; Gould *et al*., 1995; Ferre & Van Rie, 2002; Pigott & Ellar, 2007). To date, several proteins have been proposed as Bt-toxin binding proteins, such as cadherins, aminopeptidase N, alkaline phosphatase (ALP), actin, glycolipids and vacular ATP synthase (V-ATPase) subunit A (Knight *et al*., 1994; Valaitis *et al*., 1995; Lee *et al*., 1996; Luo *et al*., 1997; Gahan *et al*., 2001; McNall & Adang, 2003; Jurat-Fuentes & Adang, 2004; Xu *et al*., 2004; Griffitts *et al*., 2005; Fernandez *et al*., 2006; Jurat-Fuentes & Adang, 2006; Shitomi *et al*., 2006; Krishnamoorthy *et al*., 2007).

Proteomics is the technology for large-scale protein analysis (Hatzimanikatis *et al*., 1999; Peng & Gygi, 2001). Proteomic analyses have been used in the search for novel Bt-toxin receptors and Bt-resistance mechanisms. Combining a proteomic approach and ligand blots, McNall and

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Adang (2003) identified actin and ALP as novel Cry1Ac binding proteins in *Manduca sexta*. ALP, actin, and V-ATP synthase subunit A were also identified as Cry1Ac binding proteins in *Heliothis virescens* by Krishnamoorthy *et al*. (Krishnamoorthy *et al*., 2007). Based on proteomic analysis of the Bt-susceptible and Bt-resistant strains of *Plodia interpunctella*, Candas *et al*. (2003) suggested that concentrations of certain midgut proteins could be associated with Bt resistance.

Brush border membrane vesicles (BBMV) prepared from the apical membrane of insect midgut cells have been widely used to study receptor binding and the molecular mode of action of Cry proteins. There are several BBMV preparation methods (Wolfersberger *et al*., 1987; English & Readdy, 1989; Abdul-Rauf & Ellar, 1999). The protocol of Wolfersberger *et al*. (1987) is commonly used when sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (combined with ligand blots) is used for protein electrophoresis. However, when two-dimensional electrophoresis (2-DE) is combined with ligand blot in Bt-binding protein research, careful attention must be paid to sample preparation, as sample quantities necessary for 2-DE are higher than for SDS-PAGE. The protocol of English and Readdy (1989) is very simple, with few steps. Abdul-Rauf and Ellar (1999) modified the protocol of Wolfersberger *et al*., including a repeated resuspension and low-speed centrifugation, and used it for small insects (mosquitoes) where maximizing quantity of rendered proteins is important. Adjustments to these and other BBMV preparation methods for Bt-receptor research using 2-DE have been reported (McNall & Adang, 2003; Krishnamoorthy *et al*., 2007). In this study we chose three BBMV preparation protocols and compared them, based on the quality and quantity of *H. armigera* Bt-binding protein obtained and separated using 2-DE and ligand blots. We aim to find a BBMV preparation method yielding the maximum of protein to identify a maximum number of binding proteins, with a high resolution of separated proteins on the ligand-blotted nitrocellulose (NC) filters. Maximum protein quality and quantity are particularly important for studies of field-collected Bt-resistant insects, usually available in limited numbers and often individually analyzed.

Materials and methods

Insect rearing and midgut dissection

Midguts of *H. armigera* larvae were obtained from a colony maintained in the laboratory since 1996 (Liang *et al*., 2005). Midguts were dissected from fifth instar larvae, the peritrophic membrane and gut contents were removed and the remaining tissue was washed in an ice-cold 0.7% NaCl solution. The cleaned tissue was placed on filter paper briefly to remove excess buffer from the tissue on filter paper and weighed before storage at −80 °C.

English and Readdy (ER) membrane protein preparation

Brush border membrane vesicles were prepared following the ER protocol (English & Readdy, 1989) with minor modifications. Midguts were suspended in a 9-fold volume (w/v) of ice-cold buffer (50 mmol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 2 mmol/L Tris-HCl pH 7.4) and homogenized on ice for a 1-min cooling period until all tissues were thoroughly homogenized. Then $CaCl_2$ was added to achieve a final concentration of 10 mmol/L $CaCl_2$, after which the mixture stood on ice for 15 min. This homogenate was then centrifuged at 4 300 *g* for 10 min at 4 °C. The supernatant was collected and centrifuged at 27 000 *g* for another 10 min. The pellet was resuspended in 1 × TBS (tris buffer solution) (25 mmol/L Tris, 2 mmol/L KCl, 127 mmol/L NaCl), then aliquoted into small volumes and stored at −80 °C.

Abdul-Rauf and Ellar (ARE) membrane protein preparation

A 9-fold volume (w/v) of ice-cold buffer A (300 mmol/L mannitol, 5 mmol/L EGTA [ethylene glycol-bis(2-aminoethyl ether)-N,N,N', N'−tetraacetic acid], 17 mmol/L Tris-HCl, 1 mmol/L PMSF) was added to the midguts, homogenized on ice for a 1-min cooling period, then mixed with an equal volume of 24 mmol/L $MgCl_2$. This homogenate stood on ice for 15 min, and was then centrifuged at 2 500 *g* for 15 min at 4 °C. The supernatant was collected and stored on ice, while the pellet was resuspended in half the original volume of buffer A−$MgCl_2$ mixture. This procedure was repeated twice, centrifuging the resuspended solution and collecting the supernatant. All collected supernatants were pooled and centrifuged at 30 000 *g* for 30 min at 4 °C. The final pellet was retained, resuspended in 100 mmol/L HEPES (2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid) (pH 7.4), and the solution aliquoted into small volumes and stored at −80 °C.

Wolfersberger et al. (W) membrane protein preparation

We slightly modified the W protocol (Wolfersberger *et al*., 1987). Midguts were homogenized in a 9-fold volume of ice-cold buffer A (300 mmol/L mannitol, 5 mmol/L EGTA, 17 mmol/L Tris-HCl, 1 mmol/L PMSF) until all were thoroughly homogenized, then mixed with an equal

volume of 24 mmol/L $MgCl_2$. They were left on ice for 15 min then centrifuged at 2 500 *g* for 15 min at 4 °C. The supernatant was transferred into a new tube and centrifuged at 30 000 *g* for 30 min at 4 °C. The pellet of the second centrifugation was resuspended in half the original volume of buffer B (150 mmol/L mannitol, 2.5 mmol/L EGTA, 8.5 mmol/L Tris-HCl, 1 mmol/L PMSF) and $MgCl_2$, and then centrifuged at 2 500 *g* for 15 min at 4 °C. The resulting supernatant was transferred into a third tube and centrifuged at 30 000 *g* for 30 min at 4 °C. The final pellet was suspended in buffer C (150 mmol/L NaCl, 5 mmol/L EGTA, 1 mmol/L PMSF, 20 mmol/L Tris-HCl, 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio] propanesulfonate). This solution was aliquoted into small volumes and stored at −80 °C.

Combined membrane protein preparation

Based on the results of the three methods above, we created a combined BBMV preparation protocol integrating aspects of all three protocols.

The dissected midgut tissues were suspended in ice-cold buffer A at the ratio of 1 g of midgut tissue per 9 mL of buffer, homogenized on ice for a 1-min cooling period, then mixed with an equal volume of 24 mmol/L $MgCl_2$. The homogenate was left on ice for 15 min, and then centrifuged at 2 500 *g* for 15 min at 4 °C. The supernatant was collected and stored on ice, while the pellet was resuspended in half the original volume of buffer A−$MgCl_2$ mixture. This step was repeated twice, each time collecting the resulting supernatants. All collected supernatants were pooled and centrifuged at 30 000 *g* for 30 min at 4 °C. The resulting pellet was resuspended in buffer B (150 mmol/L mannitol, 2.5 mmol/L EGTA, 5 mmol/L Tris-HCl, 1 mmol/L PMSF), left on ice for up to 4 h, then centrifuged at 16 000 *g* for 15 min at 4 °C. The final pellet was suspended in buffer C and the resuspended solution was aliquoted into small volumes and stored at −80 °C.

BBMV were prepared three times for each protocol above, and 2-DE and ligand blots were performed twice for each prepared BBMV.

Protein quantification

All BBMV protein prepared above was quantified using the Bradford protein assay (Bradford, 1976) with BSA as the standard.

Two-dimensional electrophoresis

We followed the 2-DE protocol in the Mini-PROTEAN Tube Cell Manual (Bio-Rad Laboratories, Hercules, CA, USA) without modification. We used tube gels in the Mini-PROTEAN tube cell (Bio-Rad laboratories, Hercules, CA, USA) for isoelectric focusing (IEF). Following the manual, we cast the tube gels using the provided tube-cell module and inserted the gel tube with a reservoir or a stopper into each of the positions in the tube adaptor. We then added an equal volume of first-dimension sample buffer (8.0 mol/L urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte [Bio-Rad Laboratories, Hercules, CA, US], 0.4% Bio-Lyte 3/10 ampholyte) to the sample and incubated this at room temperature for 10 min. The mixture was added into the reservoir at the top of the gel and overlaid with 20−40 μL sample overlay buffer (4.0 mol/L urea, 0.8% Bio-Lyte 5/7 ampholyte, 0.2% Bio-Lyte 3/10 ampholyte, bromophenol blue). Electrophoresis was conducted at 500 V for 10 min, then the voltage was increased to 750 V for an additional 3.5 h. After the first round of electrophoresis, the capillary gel was pushed out from the tube with an ejector. To equilibrate the capillary gel, we submerged it in SDS sample equilibration buffer (0.0625 mol/L Tris-HCl, pH 6.8, 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol, bromophenol blue) for 10 min. Equilibrated gels were subsequently overlaid on a 10% SDS-PAGE gel. A Mini-PROTEAN 3 Cell was used for the second electrophoresis in a 10% SDS-PAGE gel. The parameters of the second electrophoresis were: 75 V for 10 min, then 150 V until the dye front reached the bottom of the gel. Separated proteins were either silver stained (Richard, 2003) or transferred to NC membrane for ligand blot.

Ligand blot

We slightly modified the Western blotting protocol in Sambrook *et al*. (1989). After BBMV were separated by 2-DE, proteins were transferred onto NC membrane blotting filters at a constant 100 V for 1 h at 4 °C. Membranes were blocked with 5% skimmed milk in PBST (137 mmol/L NaCl, 2 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4, pH 7.4, 0.05% Tween-20) for 2 h or overnight. After blocking and all incubations, filters were washed five times in PBST for 5 min per wash.

After blocking, the filter was incubated with activated Cry1Ac (0.5 μg/mL) for 2 h at room temperature or overnight at 4 °C. Rabbit antisera (provided by Chinese Academy of Sciences) against purified Cry1Ac was used as the primary antibody. The primary antibody was diluted and incubated for 2 h at room temperature. The secondary antibody was goat anti-rabbit antibody conjugated with streptavidin-horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and diluted 2 000 times. Binding proteins were visualized by 3-3'-

diaminobenzidine following the method described in Sambrook *et al*. (1989). Negative controls have been done with the absence of Cry1Ac.

Results

Effect of protein preparation procedures on BBMV quantity

Brush border membrane vesicle protein prepared with these four methods were quantified using the Bradford protein assay (Bradford, 1976). Our combined protocol yielded up to 4.8 mg proteins from 1 g of midguts. The ER, ARE and W protocols yielded 1.8, 5 and 3 g, respectively. Thus, the combined and ARE methods had the highest protein yields.

Effect of protein preparation procedures on Cry1Ac binding protein separation by 1D gel electrophoresis

Brush border membrane vesicle proteins were prepared by the four different protocols prior to 1D electrophoresis (SDS-PAGE). The BBMVs prepared with the four different methods showed similar number of protein bands by SDS-PAGE analysis, but the relative intensities of the protein bands varied among the samples prepared with the different methods (Fig. 1A). The ligand blots (Fig. 1B) showed that the Cry1Ac binding proteins from the BBMVs were in the range of 40 to nearly 200 kDa in molecular weight. Again, the number of bands was similar but the quantity of some protein bands differed between the four protocols.

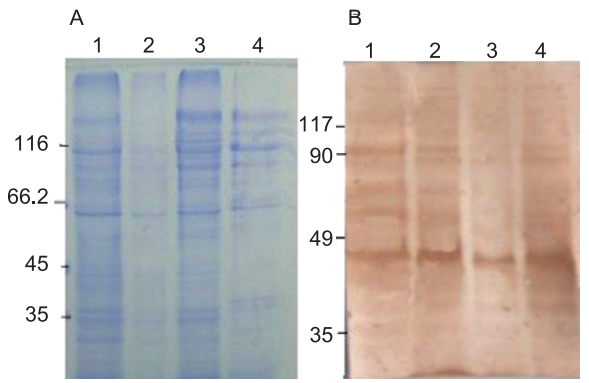

Fig. 1 SDS-PAGE (A) and ligand blot (B) of *Helicoverpa armigera* midgut brush border membrane vesicle (BBMV) proteins. For each lane 20 μg of BBMV were used. BBMV were prepared by the combined (A, lane 1; B, lane 4), Wolfersberger *et al*. (A, lane 2; B, lane 3), Abdul-Rauf and Ellar (A, lane 3; B, lane 2), and English and Readdy (A, lane 4; B, lane 1) protocols.

Effects of protein preparation procedures on Cry1Ac binding proteins separation by 2-DE

Helicoverpa armigera midgut proteins were extracted by the three published protocols prior to 2-DE. Each gel was silver-stained after electrophoresis and showed different gel features. In 2-DE most proteins were < 100 kDa, consistent with other reports (McNall & Adang, 2003; Krishamoorthy *et al*., 2007). There were some 'chains' of proteins in 2-DE, representing one protein with different degrees of post-translational modification (McNall & Adang, 2003).

More spots were obtained when protein was extracted with the ARE protocol (Fig. 2B), suggesting that this would produce the most proteins from the sample. However, the ligand blot showed similar results for all protocols, with similar kinds and numbers of binding proteins from the three protocols and negative control showed no signal (data not show). Thus, the three protocols were similarly effective for Cry1Ac-binding protein research in *H. armigera*. The ligand blots showed that our combined protocol for isolation of *H. armigera* BBMV proteins, based on the three published protocols, is suitable for 2D proteomics analysis of Cry1Ac-binding proteins (Fig. 2D).

Discussion

Brush border membrane vesicles prepared from insect midguts have proven to be an important tool to study Bt receptors. Most researchers currently isolate BBMV proteins from insect larval midguts using the protocol of Wolfersberger *et al*. (1987). However, there are other reported methods to prepare BBMV proteins, such as the ER and ARE protocols, which are not widely used. Keeton *et al*. (1998) compared three BBMV isolation methods and ligand-binding procedures to determine whether these protocols affected the toxin-binding characteristics of a common receptor BT-R_1, in *M. sexta*. The results showed that the isolation methods had no significant influence on the toxin binding to BT-R_1. SDS-PAGE has been commonly used to separate proteins in the past, but 2-DE is now more widely used. Some researchers now use proteomics to analyze Cry toxin-binding proteins (McNall & Adang, 2003; Krishnamoorthy *et al*., 2007), in these studies, different BBMV preparation methods are used. McNall and Adang (2003) used a modified ER protocol, while Krishnamoorthy *et al*. (2007) used a modified W protocol. Sample preparation is very important for 2-DE, so in the present study we compared three published BBMV isolation methods to determine the most suitable for studying *H. armigera* Cry toxin-binding proteins using proteomic

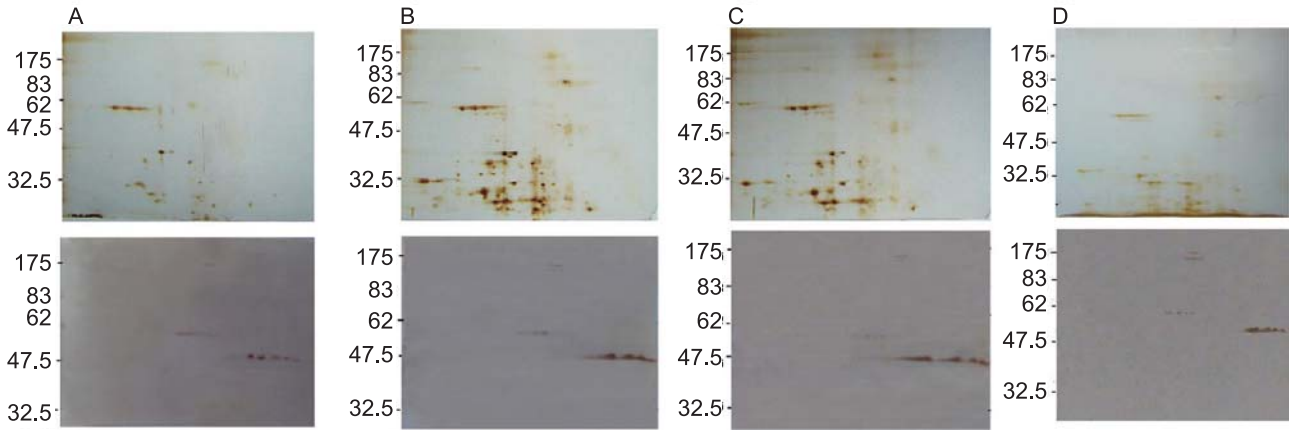

Fig. 2 Two-dimensional (2D) electrophoresis (above) and ligand blots (below) of *Helicoverpa armigera* midgut brush border membrane vesicle (BBMV) proteins. For 2-DE, 40 μg of BBMV proteins were used and for ligand blot 120 μg for each protocol. BBMV were prepared by English and Readdy (A), Abdul-Rauf and Ellar (B), Wolfersberger *et al*. (C), and the combined (D) protocols. BBMV were separated by 2-DE (above), and transferred to NC filter (below).

analysis. The three different BBMV preparation protocols (ER, ARE and W) did not affect the number or types of *H. armigera* Cry1Ac-binding proteins detected. This suggested that the different homogenized buffers and centrifuge speeds of these protocols did not affect BBMV preparation. However, different methods yielded different protein quantities. The ARE method centrifuged the resuspended solution repeatedly to obtain more protein, and was better than the other two, but had limitations. The ARE protocol had no steps to further purify proteins after high-speed centrifugation (as for the W protocol), which made the final protein solution turbid. Consequently, we combined a BBMV isolation protocol using the foresteps from the ARE protocol to maximize protein yield, and the hind part of the modified W protocol to minimize ion concentration of the protein solution. In the W protocol, half the concentration of homogenized buffer was used in the latter part to lower ion concentration in protein solution, which was important in 2-DE. In conclusion, our combined BBMV preparation protocol, based on the three previous isolation methods, does not hinder separation of the Bt receptors and can yield high quantities of protein. This is very important for studies where insect numbers are limited, such as detection for Bt resistance in field populations, and also in 2-DE because sample loading in 2D is larger than for 1D.

Isoelectric focusing (IEF) is commonly performed using products such as Protean IEF cell (Choe & Lee, 2000). IEF separations performed in tube gels using ampholytes to form the pH gradient during electrophoresis often exhibit gradient drift and the results can be variable. However, in this study we had consistent results with tube gels for IEF. We also used Immobiline DryStrips (Bio-Rad laboratories, Hercules, CA, USA) in Protean IEF cell for IEF and transferred this to NC membrane, with results that were no better than the ligand blots using tube gels (data not shown). Thus, we used Mini-PROTEAN tube cell for IEF. We followed the IEF protocol in the Mini-PROTEAN tube cell manual (Bio-Rad laboratories, Hercules, CA, USA) without modification, since the method was similarly best for the three protocols.

It was interesting that in Figure 2 the proteins on the above were not consistent with blot results on the below; we reproduced the 2-DE to ligand blots many times, with similar results. This might be due to different amounts of proteins in 2-DE and ligand blots. More proteins were used in ligand blots since 40 μg of proteins was sufficient for 2-DE with silver staining but not for visualization in blots, this suggested that the Cry1Ac binding proteins were some minor proteins in BBMV.

In this study, we did not identify the proteins detected by ligand blots, because this was sufficient to compare the three protocols, and the approximate weight and pI of proteins could be found from 2-DE and ligand blots.

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